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Certifying Officer

MS PROVISIONAL PATENT APPLICATION
PTO/SB/16(8-00)

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.53 (c).

Filing Date	October 23, 2003	Docket No.	1781-0249P

INVENTOR(s)/APPLICANT(s)

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☐ Additional inventors are being named on the separately numbered sheets attached hereto

TITLE OF THE INVENTION (280 characters max)

Fluorescence Cross-Correlation Spectometry Using a Single Laser Wavelength for Excitation and/or Dispersive Elements for Wavelength Separation for Detection

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages:* 5
☒ Drawing(s) *Number of Sheets:* 5
☐ Application Data Sheet. See 37 CFR 1.76.
☐ Other (specify):_____

METHOD OF PAYMENT *(check one)*	PROVISIONAL FILING FEE
☒ Applicant claims small entity status. See 37 CFR 1.27.	☒ Small Entity ($80.00)
☒ A check or money order is enclosed to cover the Provisional filing fees.	☐ Large Entity ($160.00)
☒ The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number 02-2448, if necessary.	

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

Date: October 23, 2003

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INVENTION DISCLOSURE FORM

Date of Submission: 4/10/2003

1. TITLE OF INVENTION

Fluorescence Cross-correlation Spectroscopy using a single laser wavelength for excitation and/or dispersive elements for wavelength separation for detection

2. DETAILED DESCRIPTION OF THE INVENTION

2.1 Field Of The Invention

- The invention relates to probes, a procedure and a device for fluorescence correlation spectroscopy, especially to multicolor crosscorrelation spectroscopy in which samples with similar excitation characteristics but different emission characteristics are excited with a single laser beam but are detected in different channels.

2.2 Background Of The Invention

- Fluorescence Correlation Spectroscopy (FCS) is a technique that can determine characteristics of molecular processes by measuring fluorescence fluctuations in a small sample volume (typically a confocal volume) that are caused by the molecular processes[1,2]. FCS uses only one fluorescent label and is limited in its resolution and can resolve two processes only when their characteristic times are different by at least a factor 2 (Ref. 3).
- Fluorescence Crosscorrelation Spectroscopy (FCCS) is a technique that allows the measurement of association events of two differently fluorescently labeled particles by detecting their distinct signals from an observation volume in at least two detectors[4,P1-P4]. The detector signals are crosscorrelated and conclusions can be drawn about the association/correlation of the two particles. This technique circumvents the limitations in resolution of FCS and can measure any kind of association independent of whether the association changes the molecular process sufficiently. E.g., the association does not have to change a molecular process (e.g. diffusion) by a factor 2 to be measured (for diffusion that means a factor 8 in mass change). However, to achieve the excitation of two fluorophores that have emission characteristics that are sufficiently different to allow separate detection of the two fluorophores, FCCS requires the use of two different lasers wavelength and thus the alignment of two laser beams to the same spot in a microscope. This procedure is difficult and has blocked the commercial and scientific exploitation of this technique[5,6].
- Recently, it was shown that FCCS can be performed with a single laser beam when two-photon excitation is used[7,P5]. The costs of the system and problems of finding fluorophores with adequate two-photon absorption cross sections are limits to this technique.
- It has been suggested that fluorophores with large Stokes' shifts can be used for simultaneous excitation with a single laser beam but no appropriate system has been suggested up to now[5,7].
- FCS and FCCS instruments are commercially available (Carl Zeiss and Olympus). However, they use two laser beams for the excitation of their samples.

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2.3 Objects Of Th Invention

- The invention proposes two fluorophore types, fluorescence energy transfer dyes[8] and semiconductor nanocrystals (quantum dots)[9] as probes with large Stokes shifts to allow the performance of FCCS with single laser beam excitation (for an example see Fig.1).
- It proposes the use of a dispersive element in the detection path to separate the emission light by wavelength and to use several detectors for the detection in distinct wavelength ranges. Calculation of the detector signals auto- and mutual cross-correlations yields information of interactions happening in a sample.
- Applications are mainly in the life sciences and especially in high-throughput screening applications (e.g. drug discovery). Discovery of interaction partners in biology, pharmacology, and medicine rely on the detection of binding events between two particles (e.g. drug and target, two interacting proteins). With conventional FCS this is only possible when the two binding partners have a mass difference of a factor 8-10. FCCS is independent of mass differences and detects specifically binding interactions. Since measurements can be done in the second range high-throughput screening is possible with this technique.
- Limitations: For the measurement of interactions both interacting partners have to be fluorescently labeled. Labeling of interaction partners can change their binding characteristics. These limitations are the same as for the conventional FCS, FCCS and other fluorescence techniques. The solution to this problem is to use the invention for lead discovery which will then be more thoroughly tested as usually done in high-throughput screening.

2.4 Summary Of The Invention

- The invention allows the measurement of FCCS with a single laser beam for excitation by using fluorophore pairs with similar excitation wavelength but different Stokes shifts to allow the excitation of the fluorophores with a single laser wavelength and the separation of the emitted fluorescence signal. We propose two large Stokes shift fluorophore types (fluorescence energy transfer yes and quantum dots) that can be used for this purpose.
- By including a dispersive element in the detection beam path and several closely spaced (ideally contiguous) detectors or an imaging detector the detected wavelength range can be controlled and different wavelength ranges can be detected simultaneously and their mutual auto- and crosscorrelations can be determined.

2.5 Brief Description Of The Drawings

- Figure 1: Normalized emission spectra of an organic dye (fluorescein-biotin, ITS Science & Medical Pte Ltd, Singapore, dotted line), a fluorescence energy transfer dye (Quantum Red, , SIGMA-Aldrich Pte Ltd, Singapore, dashed line), and a semiconductor nanocrystal (QD655, Quantum Dot Corp., CA, USA, dot-dash line). In the picture is indicated the laser excitation wavelength (a), the emission filter transmission range for detector 1 (b), the dichroic mirror center wavelength for the separation of the emission light into detector 1 and 2 (c), and the emission filter transmission range for detector 2 (d). This is an example how small Stokes shift organic dyes can be used in combination with large Stokes shift fluorescence energy transfer dyes or semiconductor nanocrystals for simultaneous excitation with a single laser wavelength in FCCS.
- Figure 2: Excitation and detection pathway for simultaneous detection of two wavelength ranges. A single laser beam is coupled over a dichroic mirror and a microscope objective in a sample. Fluorescence excited by the laser beam and emitted from the sample is spatially filtered by a pinhole. The emission light is then separated by wavelength by a dichroic mirror and detected on two different detectors, which can use optical filters to further restrict the wavelength range of detection. The light from the filters is then auto- and cross correlated.
- Figure 3: Example of an application: A binding curve (full circles) recorded for streptavidin biotin binding in which biotin was labeled with fluorescein (fluorescein-biotin) and streptavidin with a energy transfer dye (Strepatavidin Quantum Red conjugate, SIGMA-Aldrich Pte Ltd, Singapore), and a negative control (empty circles) in which binding of the fluorescence labeled molecules was suppressed by an excess of unlabeled biotin (Amersham Bisosciences Ltd., Singapore). The y-axis denotes N as defined in section 2.6 and the x-axis is the ratio of the concentration of biotin-fluorescein to quantum-red.
- Figure 4: As Fig. 2 but after passage of the pinhole, the emission light is separated according to wavelength by an imaging spectrograph (a set of lenses and a dispersive element) and detected by several detectors. From the signal of the detectors all auto- and cross-correlation functions can be calculated.
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- Fig. 5: Calculation of the ratio of N (see mathematical treatment) for the case of non-binding ($N_{negative}$) over binding ($N_{positive}$) conditions versus the dissociation constant K_d. Ligand and receptor are assumed to be labeled with fluorescein and tetrmethylrhodamine respectively. The concentration of the receptor is assumed to be 10 nM, the concentration of the ligand either 10 or 100 nM. For the calculation we measured the constants η_{11}, η_{12}, η_{21}, and η_{22} in our setup in counts per second (cps) when excited at 476 nm: η_{11}=37500 cps; η_{12}=5500 cps; η_{21}= 3400 cps and η_{22}= 6000 cps. The horizontal line at a ratio of 1.2 indicates an estimated level (from measurement errors) above which we can make a decision about differences between the binding and non-binding case. The calculations clearly indicate that the method works even with organic dye pairs that can be excited with one laser wavelength and have only small differences in their maximum emission wavelength.

2.6 Detailed Description Of The Preferred Embodiments

- Fluorophores have characteristic excitation wavelengths and emit light at a longer wavelength. The difference between the maximum excitation wavelength and maximum emission wavelength is called the Stokes shift. Up to now no fluorophore pair has been proposed that can be excited at one single laser wavelength but whose Stokes shifts are sufficiently different to allow the detection of the emission of the two fluorophores in different channels. We propose the use of: a) Fluorescence energy transfer dyes, i.e. dyes that are composed of a donor and acceptor fluorophore which can transfer excitation energy from one dye to another by a radiationless process[10]. In these dyes the donor fluorophore is excited. Since the emission spectrum of the donor is overlapping with the excitation spectrum of the acceptor energy transfer between the two dyes is possible. Emission of the fluorescence energy transfer dye takes then place at the emission wavelength of the acceptor dye. The Stokes shift of these dyes can thus reach 100 nm. b) Semiconductor nanocrystals/quantum dots can be excited at any wavelength below a certain threshold wavelength and their emission characteristics depend on their size. By choosing an excitation wavelength that is sufficiently low a large difference between excitation and emission can be achieved. Using any of these two fluorophores in combination with fluorophores of smaller Stokes shift but similar excitation characteristics allows the simultaneous excitation of both with a single laser wavelength and at the same time the detection of their particular emission wavelength in different detectors. These flurophore pairs (large and small Stokes shift fluorophore) are ideal labels for FCCS measurements that can be performed with a single laser wavelength.
- FCCS with single laser wavelength excitation was performed with the setup in Fig. 2. Results are shown in Fig. 3. A laser is coupled into a microscope by means of a dichroic mirror and focused into a sample. The large and small Stokes shift fluorophores in the sample are excited, their emission is collected by the microscope objective, passes the dichroic mirror, which separates the excitation and emission light, and is spatially filtered by a pinhole (confocal principle). The emission light is then separated by a second dichroic mirror into the distinct emissions from the large and small Stokes shift fluorophores. The detector signals are the auto- and crosscorrelated to yield information on the interaction of the fluorescently labeled particles.
- Another mode of practice of the invention is the inclusion of an imaging spectrograph (lenses and dispersive element) in the detection beam path (Fig. 4). The dispersive element separates the detected light according to wavelength. The emission light will then be detected on a range of detectors or a detector with multiple elements that are closely spaced (or contiguous). Each detector (or detector element) will measure a different part of the wavelength spectrum. The signals from the different detectors can either be treated individually or can be mutually added to create signals for larger parts of the spectrum. The resulting signals can then be autocorrelated and/or mutually cross-correlated. This invention allows the measurement of more than two distinct wavelength ranges in the emission light and has the capability of adjusting the different wavelength ranges in their extent.
- Despite apparent difficulties in finding appropriate fluorophores pairs for a FCCS system with single laser wavelength excitation[5,7], preliminary measurements and calculations show that this method can be used even for pairs of organic dyes with much smaller differences in Stokes shift (e.g. rhodamine 6G, fluorescein).

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- Mathematical treatment:

1) The crosscorrelation functions of the two detectors in FCCS are calculated by[4]:

$$G_{cross}(\tau)=\frac{\langle F_1(t)F_2(t+\tau)\rangle}{\langle F_1(t)\rangle\langle F_2(t)\rangle}$$

where $F_1(t)$ and $F_2(t)$ denote the fluorescence signals in the different detectors at time t, τ is the correlation time, and the brackets denote the time average.

Let's define the following parameters (all parameters can be measured by observing the particles in the spectrometer individually):

η_{11}: fluorescence signal of particle 1 detected in detector 1.

η_{12}: fluorescence signal of particle 1 detected in detector 2.

η_{21}: fluorescence signal of particle 2 detected in detector 1.

η_{22}: fluorescence signal of particle 2 detected in detector 2.

q_1: change of fluorescence yield of particle 1 upon binding to particle 2.

q_2: change of fluorescence yield of particle 2 upon binding to particle 1.

C_1: concentration of particles 1 which are not bound.

C_2: concentration of particles 2 which are not bound.

C_{12}: concentration complexes formed between particles 1 and 2.

V_{eff}: effective observation volume.

N_a: Avogadro's number.

Looking only at the amplitudes of the cross correlation function, i.e. at τ=0, we obtain:

$$G_{cross}(\tau)=\frac{\eta_{11}\eta_{12}C_1+\eta_{21}\eta_{22}C_2+q_1q_2(\eta_{11}+\eta_{21})(\eta_{22}+\eta_{12})C_{12}}{V_{eff}N_A(\eta_{11}C_1+\eta_{21}C_2+q_1(\eta_{11}+\eta_{21})C_{12})(\eta_{12}C_1+\eta_{22}C_2+q_2(\eta_{12}+\eta_{22})C_{12})}$$

In Fig. 3 we have depicted the value

$$N=\frac{1}{G_{cross}(\tau)-1}.$$

Depending on the interaction of particles 1 and 2 and the influence of the interaction on the fluorescence yields, N will change characteristically for a given system. The difference between the case of binding and non-binding can easily be seen in Fig. 3.

2.7 Modifications Of The Preferred Embodiments (if any)

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Figure 1

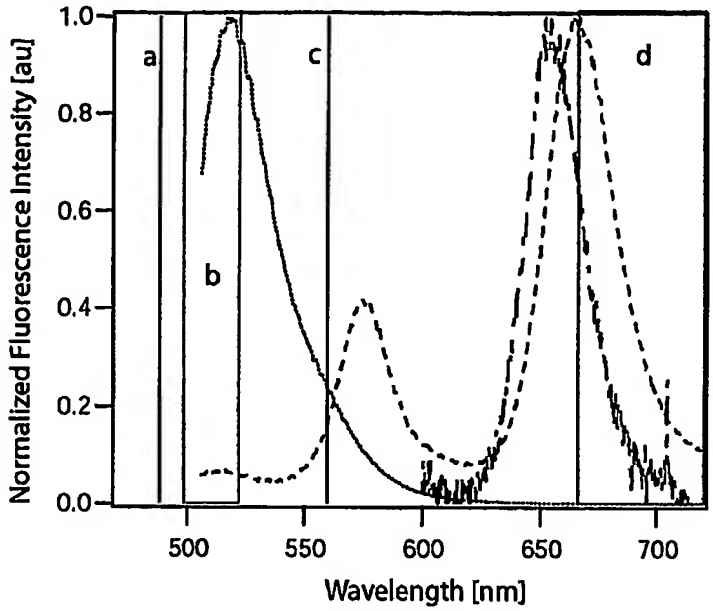

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Figure 2

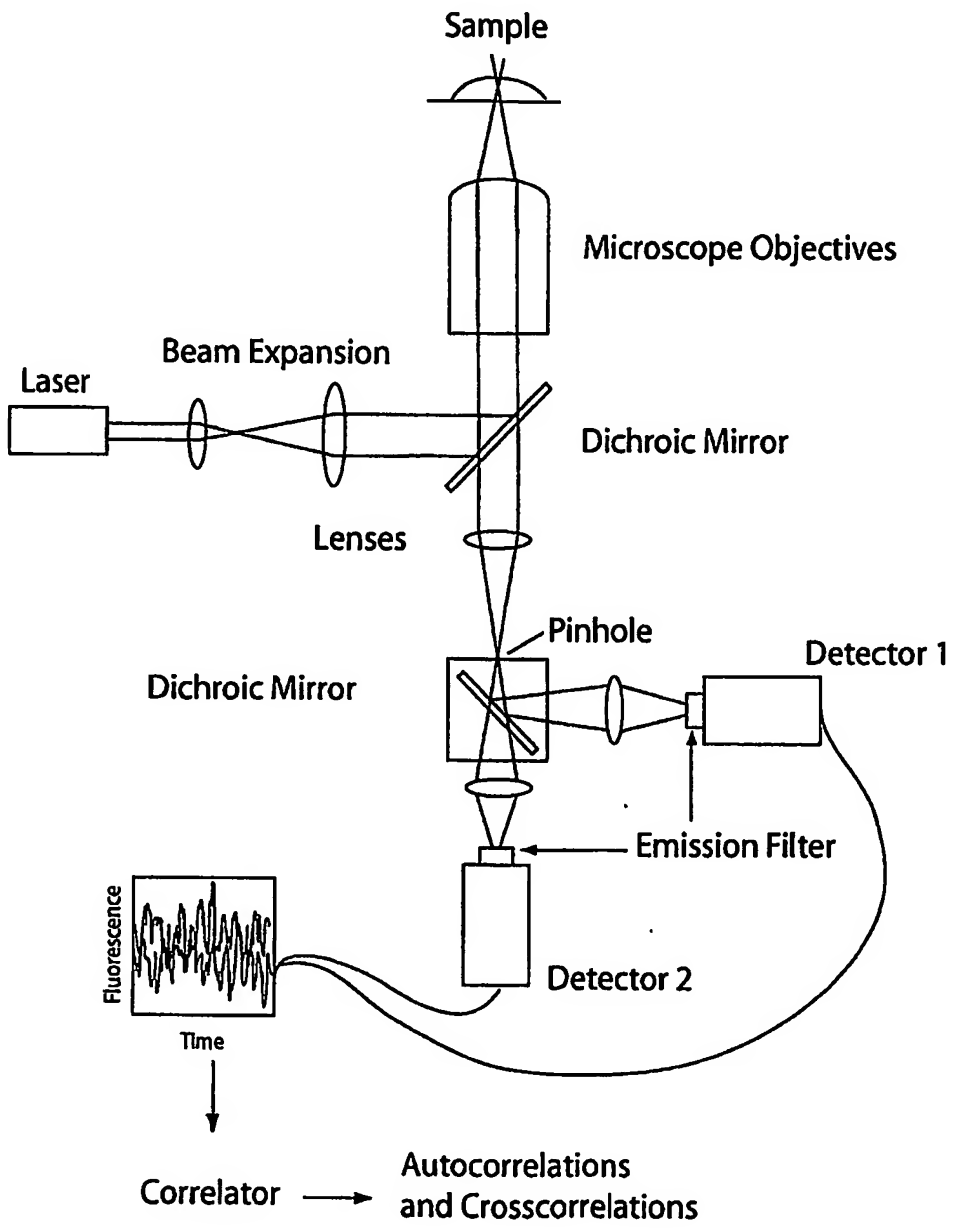

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Figure 3

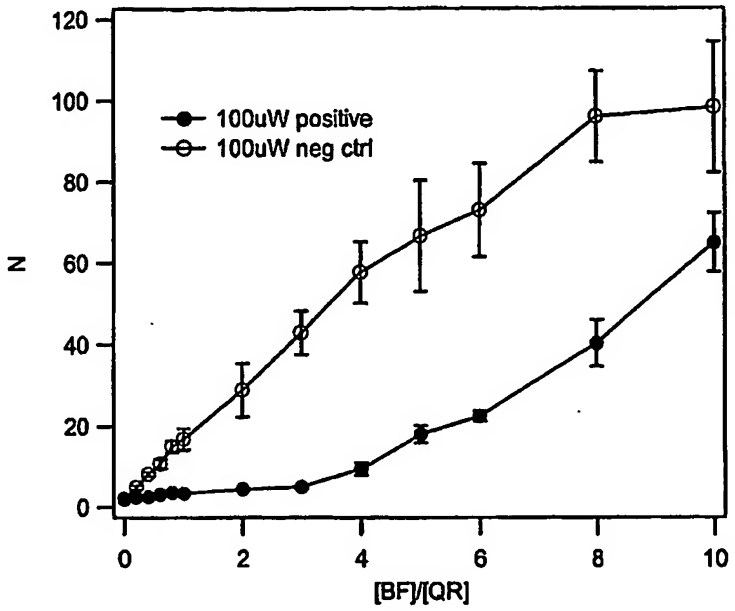

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Figure 4

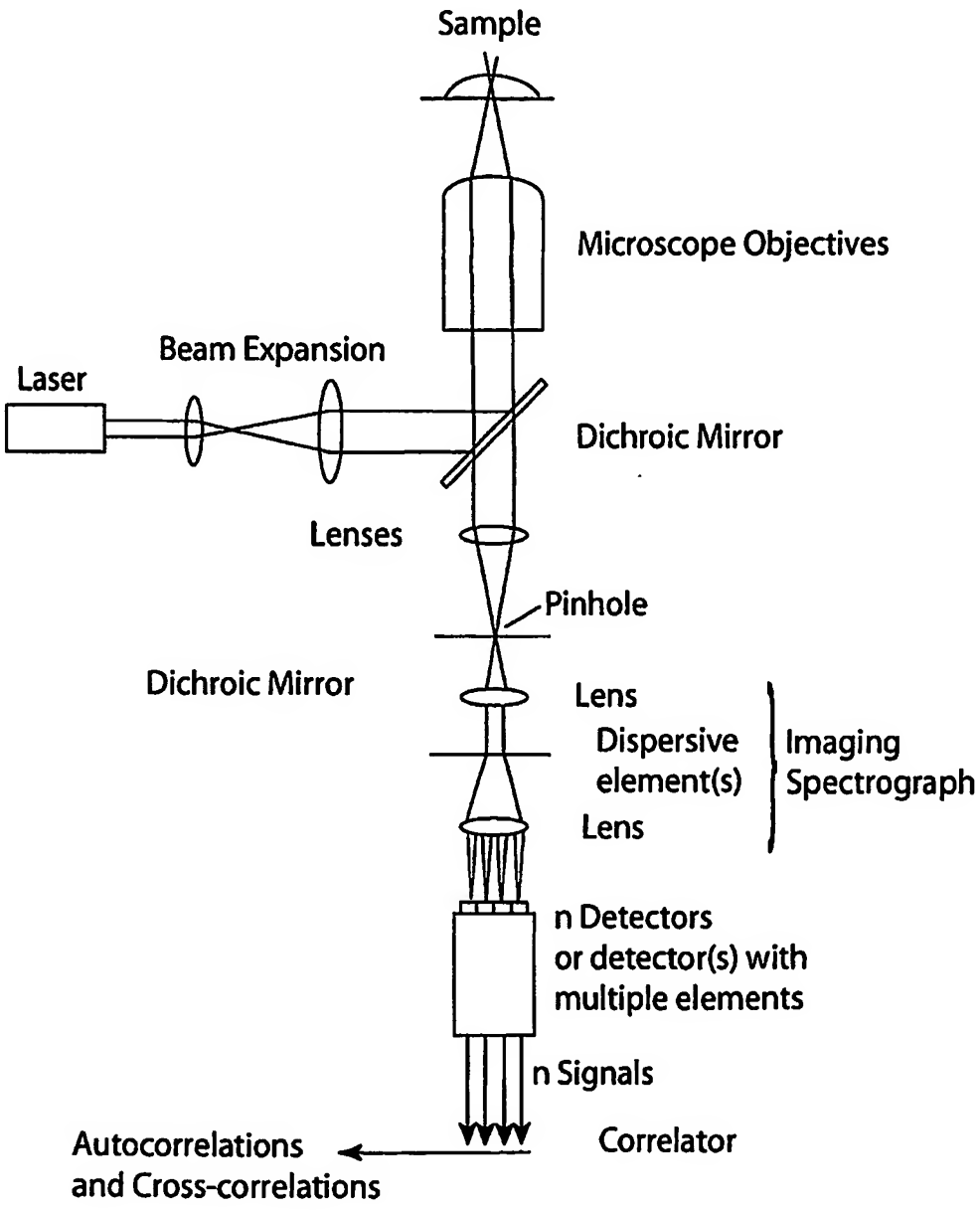

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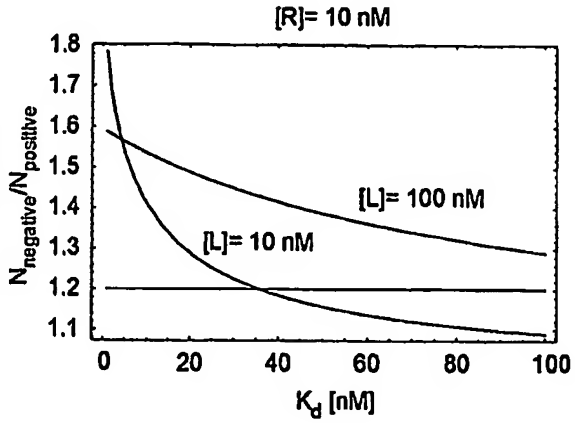
Figure 5



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2.8 References and Prior Art

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List of prior art searches (patents and non-patent literature) relevant to the invention:

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P2. US6396580; 2002-05-28; TEWES MICHAEL (DE); LANGOWSKI JOERG (DE); Method and device for polychromatic fluorescence correlation spectroscopy.

P3. DE19949658; 2001-05-10; LANGOWSKI JOERG (DE); WACHSMUTH MALTE (DE); Measurement of fluorescence cross-correlations for studying biological reactions comprises measuring correlation between fluorophore-labelled particles with different decay curves which are excited by identical spectral frequencies.

P4. WO99/34195; June 22, 2000; Eigen; Manfred (Hamburg, DE); Winkler; Thorsten (Hamburg, DE); Stephan; Jens (Hamburg, DE); Schwille; Petra (Hamburg, DE); Koltermann; Andre (Hamburg, DE); Kettling; Ulrich (Hamburg, DE); Dorre; Klaus (Hamburg, DE); Bieschke; Jan (Hamburg, DE); Method for detecting reactions by means of coincidence analysis.

P5. DE10035190; 2002-02-07; HEINZE KATRIN (DE); KETTLING ULRICH (DE); SCHWILLE PETRA (DE); KOLTERMANN ANDRE (DE) .

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